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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,220,853**, on January 16, 1998, by **HSC RESEARCH AND DEVELOPMENT LIMITED PARTNERSHIP**, assignee of Chaim M. Roifman, for "Human Lymphoid Protein Tyrosine Phosphatases".

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Human Lymphoid Protein Tyrosine Phosphatases

Field of the Invention

This invention relates to a human non-receptor protein tyrosine phosphatase (LyP1) and an isoform of LyP1, called LyP2. More particularly, it relates to the cDNA sequence of human LyP1 and LyP2, the protein products and the expression and role of these phosphatases in humans.

Background of the Invention

Protein tyrosine phosphorylation, a key mechanism of cellular signal transduction, is regulated by the action of both protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). Originally PTKs were believed to control the process of tyrosine phosphorylation, with a small number of PTPases playing largely housekeeping roles. Unexpectedly, the structural diversity of the growing number of PTPases has called this idea into question and it has become apparent that PTPases have important roles in the regulation of growth and differentiation in both normal and neoplastic cells (1,2). All PTPases contain a catalytic domain of approximately 200-300 residues including a subset of highly conserved amino acids that play a role in substrate recognition and tyrosine dephosphorylation (3). The PTPases family can be divided broadly into two major classes: membrane bound receptor like, and non-receptor like or intracellular phosphatases (4,5). Both types can be further subdivided into subfamilies based on their sequence similarities and non-catalytic domain structure motifs (6,7).

The receptor PTPases contain one or two intracellular phosphatase domains and often Ig-like domains and fibronectin-like extracellular regions (6) that play a role in cell-cell or cell-matrix interactions (8). In fact, some receptor PTPases appear to participate in homophilic and heterophilic binding interactions suggestive of a role in cell guidance and contact inhibition (7,8).

The non-receptor phosphatases display various intracellular localizations determined by amino acid sequences outside the catalytic domain (9, 35, 40). Some contain conserved non-catalytic domains such as the Src homology 2 (SH2) and SH3 domains allowing them to interact with a variety of tyrosine phosphorylated proteins

and proteins containing proline rich sequences (10, 11). Cytoplasmic PTPases have been found associated with a variety of PTKs including CSK and the Jak kinases, and a number of cytokine and antigen receptors (7, 11,31).

Several lines of evidence indicate that within the immune system, PTPases are essential for lymphocyte development and activation. CD45, a transmembrane phosphatase expressed exclusively in hematopoietic cells (12), is required for antigenic activation of B and T lymphocytes (13, 14). In addition, evidence from CD45-deficient mice indicates that CD45 also plays a pivotal role in thymic development and T cell apoptotic response to T cell receptor engagement (15, 16). Recent studies have suggested that the hematopoietic-specific intracellular phosphatase-SHP1 (SH2 containing PTPase) negatively regulates signaling through association with the B cell receptor, PcγRIIB1 (17) and the IL-3 receptor 3 chain (18). SHP1 also participates in T cell signalling events through dephosphorylation of the T cell receptor (TCR), $p56^{lck}$ and ZAP-70 (19). Mutations in the murine *motheaten* locus coding the SHP1 protein result in severe combined immunodeficiency and systemic autoimmunity, as well as many other hematopoietic abnormalities (20). Furthermore, expression of HePTP, a cytoplasmic hematopoietic specific PTPase, is induced in lymphocytes stimulated by phytohemagglutinin, concavalin A, lipopolysaccharide and anti-CD3 (21), suggestive of a role in lymphocyte activation pathways. Together, these studies suggest a critical role for phosphatases in the development of the immune system.

Summary of the Invention

A human gene encoding a non-receptor phosphatase predominantly expressed in lymphoid cells has been identified and designated Lymphoid Protein Tyrosine Phosphatase (*LyP1*) gene. In addition, an isoform of *LyP1* has also been identified and designated *LyP2*. This isoform is a product of C-terminal alternative RNA splicing resulting in a protein which is 116 amino acids shorter than LyP1 and contains an alternative C-terminal of seven amino acids. The LyP gene is located on chromosome 1p13. The cDNA sequences encoding for LyP1 and LyP2 have been cloned, sequenced and expressed to provide the respective proteins. The proteins are

most highly expressed in lymphoid tissues including spleen, lymph nodes, peripheral leukocytes, tonsil B and T lymphocytes and in bone marrow.

In accordance with one embodiment of the invention, an isolated nucleic acid is provided comprising a nucleotide sequence encoding human LyP1 protein. The nucleic acid may be in the form of DNA, genomic DNA, cDNA, mRNA and various fragments and portions of the sequence encoding human LyP1.

In accordance with another embodiment of the invention, an isolated human gene which maps to chromosome 1p13 and encodes human LyP1 protein.

In accordance with another embodiment of the invention, an isolated human gene which maps to chromosome 1p13 and encodes human LyP2 protein.

In accordance with a further embodiment of the invention, a purified nucleotide sequence is provided comprising genomic DNA, cDNA, mRNA, anti-sense DNA or homologous DNA corresponding to the cDNA sequence of Sequence ID No.1.

In accordance with a further embodiment of the invention, is a purified nucleotide sequence sharing 95% homology to Sequence ID No.1 and encoding human LyP1 protein.

In accordance with a further embodiment of the invention is a substantially pure human LyP1 protein. In accordance with a further embodiment is a protein comprising the amino acid sequence of Sequence ID No.2.

In accordance with another embodiment of the invention are protein fragments comprising at least 12 contiguous amino acids of Sequence ID No.2.

In accordance with a further embodiment of the invention is a purified protein sharing 95% homology to Sequence ID No.2 and having human LyP1 activity.

In accordance with a further embodiment of the invention, a substantially pure polypeptide is provided comprising at least one functional domain of a human LyP1 protein.

In accordance with a further embodiment of the invention, a substantially pure polypeptide is provided comprising an antigenic determinant of a human LyP1 protein.

In accordance with another embodiment of the invention, an isolated nucleic acid is provided comprising a nucleotide sequence encoding human LyP2 protein.

The nucleic acid may be in the form of cDNA or mRNA and various fragments and portions of the gene sequence encoding human LyP2.

In accordance with a further embodiment of the invention, a purified nucleotide sequence is provided comprising cDNA, mRNA, anti-sense DNA or homologous DNA corresponding to the cDNA sequence of Sequence ID No.3.

In accordance with a further embodiment of the invention, is a purified nucleotide sequence sharing 95% homology to Sequence ID No.3 and encoding for human LyP1 protein.

In accordance with a further embodiment is a substantially pure human LyP2 protein.

In accordance with a further embodiment of the invention is an amino acid sequence corresponding to the amino acid sequence of Sequence ID No.4.

In accordance with a further embodiment of the invention, a method is provided for altering the lymphoid cell differentiation pathway in which LyP1 and/or LyP2 is active comprising administering to the cell an agent selected from the group consisting of;

(a) monoclonal or polyclonal antibodies specifically binding human LyP1 or LyP2 protein;

(b) a polynucleotide antisense strand capable of hybridizing to human LyP1 and gene or gene transcripts;

(c) an agent to alter phosphorylation of human LyP1or LyP2 protein;

(d) a composition comprising a mixture of (a) to (c).

In accordance with a further embodiment of the invention, a method is provided for identifying allelic variants or heterospecific homologues of a LyP1 gene comprising

(a) choosing a nucleic acid probe or primer capable of hybridizing to a human LyP1 gene sequence under stringent hybridization conditions;

(b) mixing said probe or primer with a sample of nucleic acids which may contain a nucleic acid corresponding to the variant or homologue;

(c) detecting hybridization of the probe or primer to the nucleic acid corresponding to the variant of homologue.

In accordance with a further embodiment of the invention, a method is provided for producing antibodies which selectively bind to a human LyP1 or LyP2 protein comprising the steps of

administering an immunogenically effective amount of a human LyP1 or LyP2 immunogen to an animal;

allowing the animal to produce antibodies to the immunogen; and

obtaining the antibodies from the animal or from a cell culture derived therefrom.

In accordance with a further embodiment of the invention, a substantially pure antibody is provided which binds selectively to an antigenic determinant of a human LyP1 or LyP2 protein.

In accordance with a further embodiment of the invention, a method is provided for identifying compounds modulating expression of a human LyP1 gene comprising;

contacting a cell with a test candidate wherein the cell includes a regulatory region of a human LyP1 gene operably joined to a coding region; and

detecting a change in expression of the coding region.

In accordance with a further embodiment of the invention, a method is provided for altering T cell differentiation in lymphoid tissues, comprising administering to the subject a therapeutically effective amount of an agent selected from the group consisting of:

an isolated antisense nucleotide sequence which hybridizes with human LyP1 or LyP2 gene sequence; and

a substantially pure monoclonal antibody which recognizes LyP1 or LyP2 protein.

In accordance with a further embodiment of the invention, a pharmaceutical composition is provided comprising an active ingredient selected from the group consisting of:

an antisense sequence which hybridizes to a human LyP1 nucleotide sequence or to a transcript of the sequence;

a substantially pure antibody which binds selectively to human LyP1 or LyP2 protein and a pharmaceutically acceptable carrier;

a mimetic of human LyP1 or LyP2 protein;

a functional analog of human LyP1 or LyP2 protein;

an inhibitor of human LyP1 or LyP2 protein activity; and

an agent capable of altering the phosphorylation state of human LyP1 or LyP2 protein.

In accordance with a further embodiment of the invention, a method is provided of screening for an agent useful in treating a disorder characterized by an abnormality in a phosphorylation signaling pathway of lymphoid cells, wherein the pathway involves an interaction between a human LyP1 or LyP2 protein and a human LyP1 or LyP2 activator, comprising screening potential agents for ability to disrupt or promote the interaction as an indication of a useful agent.

In accordance with a further aspect of the invention, a method is provided of preventing or treating a disorder in a mammal characterized by an abnormality in an intracellular phosphorylation signaling pathway of lymphoid cells, wherein the pathway involves an interaction between a human LyP1 or LyP2 protein and a human LyP1 or LyP2 substrate, comprising the step of disrupting or promoting said interaction *in vivo*.

In accordance with another aspect of the invention, a transgenic mouse model for various diseases involving abnormally activated lymphoid cells which comprises an animal which has in its genome a human gene encoding LyP mutated to manifest altered activation. The transgenic mouse may exhibit symptoms indicative of lymphoid cell disorders.

In accordance with another aspect of the invention, the LyP1 or LyP2 protein can be used as a starting point for rational drug design to provide ligands, therapeutic drugs, inhibitors or other types of small chemical molecules.

Brief Description of the Drawings

Certain embodiments of the invention are described, reference being made to the accompanying drawings, wherein:

Figure 1 shows a Schematic diagram of LyP1 and LyP2 deduced from the cDNA clones. Boxes indicate the open reading frame, with thin lines representing the 5' and 3' untranslated regions. The six overlapping cDNA clones (bold black lines)

obtained from a human thymus cDNA library are shown under the schematic structures of the cDNAs.

Figure 2 shows an alignment of LyP1 and Z70PEP amino acid sequences. The PTPase domain is indicated by brackets. An arrow indicates the end of the amino acid sequence shared by LyP1 and LyP2 and the beginning of the unique C-terminal sequence of LyP1. The NXXY motif is indicated by line above the sequence. The four potential SH3 domain binding sites are also indicated (asterisks). A consensus sequence is shown below the alignment.

Figure 3 demonstrates that LyP2 is a result of alternative splicing of the LyP1 gene. A, schematic map of the PCR strategy used. Primer 1 corresponds to the last 20 nucleotides shared by both LyP1 and LyP2 sequences, primer 2 to LyP2 untranslated area and primer 3 to the beginning of the novel LyP1 sequence, immediately downstream of primer 1. (see also C). B. The results of the PCT amplification on genomic DNA. Lane 1, DNA size markers, Lane 2, a product of 3.5kb was amplified with primers 1 and 3. Lane 3, is a product of 100bp was amplified with primers 1 and 2. C. Schematic map of LyP1 splicing. The sequences before the vertical line represent the splice donor site, while the nucleotide sequences after it are the LyP1 intronic sequence which code for the unique C-terminal seven amino acids, stop codon (asterisk) and untranslated sequence (lower case letters) of LyP1. A white box represents the common cDNA sequence shared by LyP1 and LyP2, the black and the light gray boxes representing the unique sequences of each cDNA (LyP1 and LyP2 respectively).

Figure 4 shows the expression profile of LyP1 transcripts. The size of RNA markers are indicated in kb. A. Human tissues of various origin. B. Immune relevant human tissues.

Figure 5 shows *in vivo* translation of LyP1 and LyP2 proteins, COS-7 cells were transiently transfected with HA-tagged LyP1 and LyP2 cDNAs and protein expression analyzed by Western blotting with anti-HA tag antibodies. Lane 1, pCDNA3. Lane 2, pCDNA3 LyP1. Lane 3, pCDNA3 LyP2. Molecular mass markers are shown in kDa.

Figure 6 shows the relative quantification of LyP1 and LyP2 transcripts in thymocytes cDNA by competitive PCR. Different concentration of competitor DNA

were added to fixed amount of sample cDNA. The results of PCR amplification products of :A. 26 cycles with specific primers to LyP1 B.35 cycles with specific primers to LyP2. The internal control concentrations are indicated below the pictures in Pico-Molar.

Figure 7 shows the localization of LyP1 and LyP2 in transiently transfected COS-7 cells by immunofluorescence. COS-7 cells were transiently transfected with HA-tagged LyP1 and LyP2 cDNAs and Immunofluorescence was performed using a monoclonal antibody against HA tag. Magnification 1000X. A. Cells transfected with HA-LyP2 cDNA. B. Cells transfected with HA-LyP1 cDNA.

Figure 8 shows regional mapping of the LyP gene by fluorescence in situ hybridization to normal human lymphocyte chromosomes counterstained with DAPI. Biotinylated cDNA probe was detected with avidin-fluorescein isothiocyanate (FITC). Part of a representative metaphase preparation is shown to indicate the position of the LyP probe FISH signals visible as two yellow fluorescent spots on the p arm of chromosome 1. A DAPI banded chromosome 1 together with schematic ideogram is shown to indicate that the LyP1 probe hybridizes to band 1p13.

Desscription of the Invention

Two novel intracellular protein tyrosine phosphatase cDNA sequences have been isolated from a human thymus cDNA library, LyP1 (Table One) and its splice variant, LyP2 (Table Two). The amino acid sequences of LyP1 and LyP2 are shown in Tables 3 and 4, respectively. Sequence analysis of LyP1 reveals significant homology with the murine phosphatase Z70PEP, an intracellular PTPase widely expressed in hematopoietic tissues (10). LyP1 shares an overall 70% amino acid identity with Z70PEP (Fig. 2). While there is 89% identity between the catalytic domain of LyP1 and Z70PEP, significantly less homology is observed within the non catalytic portion (61%), which clearly contains a large area of unique sequence. Within this area LyP, but not Z70PEP, contains an NXXY sequence (see Fig. 2); when tyrosine phosphorylated this motif may be recognized by a phosphotyrosine binding (PTB) domain (29). PTB domains are found in several signal transduction adaptor proteins (eg, IRS-1, Shc), while the NXXY motif is found in several receptor tyrosine kinases and signaling pathway proteins (29,30). LyP1 also contains four

proline rich sequences (see Fig 2), forming putative PXXP and class II (XPPLPXR) SH3 domain binding motifs (31). Indeed, a recent study has demonstrated association of a proline-rich motif (PPPLPERTP) of the closely related phosphatase, Z70PEP with the SH3 domain of the protein tyrosine kinase $p50^{CSK}$ (32).

The LyP1 ORF codes for a protein of 808 amino acids. Hydropathy analysis indicates that LyP1 contained no obvious signal sequence or hydrophobic segments and is therefore predicted to be an intracellular protein. The N-terminal region of the LyP1 amino acid sequence contains a single phosphatase catalytic domain. Sequence analysis revealed 70% overall amino acid identity between LyP1 and the murine phosphatase Z70PEP (89% within the catalytic domain and 61% within the non-catalytic portion); while the homology between the phosphatase domain of LyP1 and other PTPases murine varies between 30 and 60%. On the basis of this analysis it was initially believed that LyP1 may be a human homologue of the murine phosphatase Z70PEP, however, data presented herein suggests that LyP1 represents a novel member of this phosphatase family.

The murine Z70PEP also possesses several consensus PEST sequences (hence its name [PEST]-domain Phosphatase) of as yet unclear function (10). PEST sequences contain an unusually high percentage of proline (P), glutamic/aspartic acid (E/D), serine (S), and threonine(T) residues and have been identified in many proteins and were proposed to confer a high susceptibility to rapid degradation (33). Early studies on the rapidly degraded enzyme orinithine decarboxylase provided support for this hypothesis (34), but such claim could not be substantiated for Z70PEP (35) or for other PEST containing proteins (36,37). An analysis of the LyP1 sequence using the program PEST-FIND (PC analysis software; Oxford Molecular Group, Oxford) indicated the presence of only a single PEST region (amino acids 702-736), while five were confirmed in Z70PEP. Thus despite the relatively high degree of homology between the catalytic domain of LyP1 and Z70PEP, significant differences between the proteins emerge upon comparison of the other domains. In view of these results it is likely that LyP1 is closely related to Z70PEP.

The pattern of LyP1 expression observed by Northern blotting suggests that it is preferentially expressed in lymphoid cells (Fig. 4), particularly in thymocytes and mature B and T cells. A low level of LyP1 expression was also seen in tissues rich in

lymphoid infiltrates, such as the small intestine and appendix. This pattern of expression suggests that LyP1 may play a role in the regulation of aspects of both early and late states of T cell differentiation. The lack of expression in fetal liver tissue, which contains a large population of pre-B cells, may suggest a different role in the biology of B cell development. The mRNA expression of LyP1 and its isoform, LyP2, was differentiated by the use of more specific probes. Lymphoid mRNAs hybridized with a probe specific for the unique C-terminal of LyP1 revealed the same pattern of expression seen in Northern blots obtained by using a cDNA fragment common to both LyP forms (not shown). This suggests that LyP2 expression is extremely low, below the threshold of detection of Northern blotting. This suggestion was confirmed by semi-quantitative PCR comparison of LyP1 and LyP2 expression. In thymocytes the expression of LyP1 was found to be 100 fold greater than LyP2 (Fig. 7). Similar results were obtained from other lymphoid cells (not shown).

LyP2 appears to be derived from alternative splicing of an intronic sequence. A 3.5kb intronic sequence of LyP1 was found to contain an alternative exon, coding for the C-terminal 7 amino acids of LyP2, and at least part of its 3' untranslated area (Fig.3). Consequently the 116 amino acid C-terminal of LyP1 is replaced by 7 alternative amino acids in LyP2. Several studies have shown that such C-terminal diversity in cytophasmic PTPases can be generated by alternative splicing. The intracellular Drosophila PTPases gene DPTP61F produces two proteins with distinct COOH-terminal sequences (38), which determine the targeting of the alternative protein forms to either a cytoplasmic or nuclear compartment. Similarly, alternative splicing produces four forms of PTP-S (S1-S4), a variant of the T cell phosphatase 39). These alternative forms perform significantly different functions, PTP-S2 and PTP-S4 demonstrating inherent differences in their substrate specificity, potential to bind DNA, and subcellular location; despite the fact that only the last 6 amino acids of PTP-S2 are replaced by an alternative C-terminal (34aa) in PTP-S4 (40). Thus, the creation of C-terminal diversity by alternative splicing in phosphatases appears to be a general mechanism of generating functional diversity.

The differences between the LyP1 and LyP2 C-terminal sequences suggested that potentially, these phosphatases could be also directed to different subcellular compartments. However, through immunofluorescent staining of transiently

transfected Cos-7 cells, it was determined that both LyP1 and LyP2 show a similar pattern of diffuse cytophasmatic staining (Fig. 7). Interestingly, there is some question about the cellular localization of the closely related murine phosphatase, Z70PEP. While it has been demonstrated that Z70PEP localized to the nucleus in transfected Hela cells, (35) it has also been demonstrated that Z70PEP localized outside the nucleus when transiently overexpressed in Cos-1 and B1-141 T cells (32).

The significance of the alternative C-terminal sequences of LyP1 and LyP2 remains unclear, but there are several differences between the C-terminal tails that may be key in revealing functional divergence. The C-terminus of LyP1, but not LyP2, contains a consensus sequence XS/TPXK/R (^{741}KTPGK ^{745}) recognized by the p34^{cdc2} kinase (41), a cell cycle regulatory kinase (42), suggesting that LyP1 may be phosphorylated in a cell cycle dependent manner. LyP1 also contains four potential SH3 domain binding sites, compared to a single motif in LyP2; suggesting the isoforms may interact with different sets of SH3 domains. Such differences, together with the major differences in the level of RNA expression, may point to participation in separate signaling pathways or alternatively, to differences in the regulation of their activity.

The expression of LyP1 and LyP2 protein in lymphoid tissues suggests it may be of functional importance to these cells, perhaps in functional differentiation of T cells and therefore these proteins may play a role in certain disorders of lymphoid origin namely, leukemia and auto immune disorders.

Using FISH analysis, the LyP gene was found to be localized to chromosome 1p13 (Fig. 8). This region is of particular interest because it is a common site of chromosomal rearrangement in both solid and hematopoietic cancer (47, 48). One such chromosomal rearrangement is a frequent alteration in the 1p13 region in chromosomally aberrant clones isolated from both patients with Hodgdin's (49, 50) and non Hodgkin's (51) lymphomas. Several lines of evidence already suggest that PTPases may act as a tumour suppression gene (2, 52). This raises the possibility of an association between an abnormality of the 1p13 locus in these patients and an alteration of LyP.

In another embodiment of the present invention expression of the LyP gene in heterologous cell systems can be used to demonstrate structure-function relationships

as well as provide for cell lines for the purposes of drug screening. Ligating the LyP DNA sequence into a plasmid expression vector to transfect cells is a useful method to test the proteins influence on various cellular biochemical parameters including the identification of substrates as well as activators and inhibitors of the phosphatase. Plasmid expression vectors containing either the entire, or portions thereof, LyP1 or LyP2 can be used in *in vitro* mutagenesis experiments which will identify portions of the protein crucial for regulatory function.

The DNA sequence can be manipulated in studies to understand the expression of the gene and its product, to achieve production of large quantities of the protein for functional analysis, for antibody production, and for patient therapy. The changes in the sequence may or may not alter the expression pattern in terms of relative quantities, tissue-specificity and functional properties. Partial or full-length DNA sequences which encode for the LyP1 and LyP2 protein, modified or unmodified, may be ligated to bacterial expression vectors. *E. coli* can be used using the T7 RNA polymerase/promoter system using two plasmids or by labeling of plasmid-encoded proteins, or by expression by infection with M13 Phage mGPI-2. *E. coli* vectors can also be used with Phage lamba regulatory sequences, by fusion protein vectors (eg. lacZ and trpE), by maltose-binding protein fusions, and by glutathione-S-transferase fusion proteins.

Alternatively, the LyP1 or LyP2 protein can be expressed in insect cells using baculoviral vectors, or in mammalian cells using vaccinia virus. For expression in mammalian cells, the cDNA sequence may be ligated to heterologous promoters, such as the simian virus (SV40) promoter in the pSV2 vector and introduced into cells, such as COS cells to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin and mycophoenolic acid.

The normal LyP DNA sequence can be altered using procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence alteration with the use of specific oligonucleotides together with PCR.

The cDNA sequence or portions thereof, or a mini gene consisting of a cDNA

with an intron and its own promoter, is introduced into eukaryotic expression vectors by conventional techniques. These vectors permit the transcription of the cDNA in eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. The endogenous LyP gene promoter can also be used. Different promoters within vectors have different activities which alters the level of expression of the cDNA. In addition, certain promoters can also modulate function such as the glucocorticoid-responsive promoter from the mouse mammary tumor virus.

Some of the vectors listed contain selectable markers or neo bacterial genes that permit isolation of cells by chemical selection. Stable long-term vectors can be maintained in cells as episomal, freely replicating entities by using regulatory elements of viruses. Cell lines can also be produced which have integrated the vector into the genomic DNA. In this manner, the gene product is produced on a continuous basis.

Vectors are introduced into recipient cells by various methods including calcium phosphate, strontium phosphate, electroporation, lipofection, DEAE dextran, microinjection, or by protoplast fusion. Alternatively, the cDNA can be introduced by infection using viral vectors.

Eukaryotic expression systems can be used for many studies of the LyP gene and gene product(s) including determination of proper expression and post-translational modifications for full biological activity, identifying regulatory elements located in the 5' region of the LyP gene and their role in tissue regulation of protein expression, production of large amounts of the normal and mutant protein for isolation and purification, to use cells expressing the LyP1 or LyP2 protein as a functional assay system for antibodies generated against the protein or to test effectiveness of pharmacological agents, or as a component of a signal transduction system, to study the function of the normal complete protein, specific portions of the protein, or of naturally occurring and artificially produced mutant proteins.

Using the techniques mentioned, the expression vectors containing the LyP1 or LyP2 cDNA sequence or portions thereof can be introduced into a variety of mammalian cells from other species or into non-mammalian cells.

The recombinant cloning vector, according to this invention, comprises the

selected DNA of the DNA sequences of this invention for expression in a suitable host. The DNA is operatively linked in the vector to an expression control sequence in the recombinant DNA molecule so that LyP1 or LyP2 protein can be expressed. The expression control sequence may be selected from the group consisting of sequences that control the expression of genes of prokaryotic or eukaryotic cells and their viruses and combinations thereof. The expression control sequence may be selected from the group consisting of the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of the fd coat protein, early and late promoters of SV40, promoters derived from polyoma, adenovirus, retrovirus, baculovirus, simian virus, 3-phosphoglycerate kinase promoter, yeast acid phosphatase promoters, yeast alpha-mating factors and combinations thereof.

The host cell which may be transfected with the vector of this invention may be selected from the group consisting of *E.coli, pseudomonas, bacillus subtillus, bacillus stearothermophilus,* or other bacili; other bacteria, yeast, fungi, insect, mouse or other animal, plant hosts, or human tissue cells.

In situ hybridization is a method used to detect the expression of LyP1 and LyP2 protein. *In situ* hybridization relies upon the hybridization of a specifically labelled nucleic acid probe to the cellular RNA in individual cells or tissues. Therefore, it allows the identification of mRNA within intact tissues, such as the brain. In this method, oligonucleotides corresponding to unique portions of the LyP gene are used to detect specific mRNA species in the brain.

In this method a rat is anesthetized and transcardially perfused with cold PBS, followed by perfusion with a formaldehyde solution. The brain or other tissues is then removed, frozen in liquid nitrogen, and cut into thin micron sections. The sections are placed on slides and incubated in proteinase K. Following rinsing in DEP, water and ethanol, the slides are placed in prehybridization buffer. A radioactive probe corresponding to the primer is made by nick translation and incubated with the sectioned brain tissue. After incubation and air drying, the labeled areas are visualized by autoradiography. Dark spots on the tissue sample indicate hybridization of the probe with brain mRNA which demonstrates the expression of the protein.

Transgenic Animal Models

The creation of transgenic animal models for abnormal lymphoid function characterized by altered LyP1 or LyP2 activity is important to the understanding of the function of these phosphatases in intracellular signaling and for the testing of possible therapies for abnormal T cell activation and differentiation. In general, techniques of generating transgenic animals are widely accepted and practiced. A laboratory manual on the manipulation of the mouse embryo, for example, is available detailing standard laboratory techniques for the production of transgenic mice (41).

There are several ways in which to create an animal model in which the LyP gene is expressed. One could simply generate a specific mutation in the mouse LyP gene as one strategy. Secondly a wild type human LyP gene and/or a humanized murine gene could be inserted into the animals genome by homologous recombination. It is also possible to insert a mutant (single or multiple) human gene as genomic or minigene construct using wild type or mutant or artificial promoter elements. More commonly, and most preferred in the present invention, knock-out of the endogenous murine genes may be accomplished by the insertion of artificially modified fragments of the endogenous gene by homologous recombination. The modifications include insertion of mutant stop codons, the deletion of DNA sequences, or the inclusion of recombination elements (lox p sites) recognized by enzymes such as Cre recombinase. Gene knockout produces homozygous mutant mice, which show symptoms or phenotype similar to those exhibited by a human.

In general, for gene knock-out, embryonic stem cells heterozygous for a knockout mutation in a gene of interest (ie. LyP gene) and homozygous for a marker gene (eg. coat colour) are transplanted into the blastocoel cavity of 4.5 day embryos homozygous for an alternate market. The early embryos then are implanted into a pseudopregnant female. Some of the resulting progeny are chimeras. Chimeric mice then are backcrossed. Intercrossing will eventually produce individuals homozygous for the disrupted allele that is, knockout mice. (Capecchi, MR. 1989. Science. 244:1299-1291).

To inactivate the LyP mouse gene chemical or x-ray mutagenesis of mouse gametes, followed by fertilization, can be applied. Heterozygous offspring can then be identified by Southern blotting to demonstrate loss of one allele by dosage, or

failure to inherit one parental allele using RFLP markers.

To create a transgenic mouse, a mutant or normal version of the human LyP gene can be inserted into a mouse germ line using standard techniques of oocyte microinjection or transfection or microinjection into stem cells. Alternatively, if it is desired to inactivate or replace the endogenous LyP gene, homologous recombination using embryonic stem cells may be applied.

For oocyte injection, one or more copies of a mutant or normal LyP gene can be inserted into the pronucleus of a just-fertilized mouse oocyte. This oocyte is then reimplanted into a pseudo-pregnant foster mother. The liveborn mice can then be screened for integrants using analysis of tail DNA for the presence of human LyP gene sequences. The transgene can be either a complete genomic sequence injected as a YAC or chromosome fragment, a cDNA with either the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

Retroviral infection of early embryos can also be done to insert the human LyP1 gene. In this method, the human LyP gene is inserted into a retroviral vector which is used to directly infect mouse embryos during the early stages of development to generate a chimera, some of which will lead to germline transmission.

Homologous recombination using stem cells allows for the screening of gene transfer cells to identify the rare homologous recombination events. Once identified, these can be used to generate chimeras by injection of mouse blastocysts, and a proportion of the resulting mice will show germline transmission from the recombinant line. This methodology is especially useful if inactivation of the human LyP gene is desired. For example, inactivation of the LyP gene can be done by designing a DNA fragment which contains sequences from a LyP exon flanking a selectable marker. Homologous recombination leads to the insertion of the marker sequences in the middle of an exon, inactivating the LyP gene. DNA analysis of individual clones can then be used to recognize the homologous recombination events.

This embodiment of the invention has the most significant commercial value as a mouse model for abnormal lymphoid cell activity and this may include disorders such as leukemia and autoimmune disorders..

Screening for Disease

In another embodiment of the invention the knowledge of the human LyP sequence provides for screening for various diseases involving abnormally activated or inactivated LyP1 or LyP2 in which the activity defect is due to a mutant LyP gene. Such defects may include, for example, leukemia which is associated with 22 known gene translocations one of which may involve mutated LyP. Other defects may include autoimmune disorders such as rheumatoid arthritis.

People with at a risk for a lymphoid disease or, individuals not previously known to be at risk, or people in general may be screened routinely using probes to detect the presence of a mutant LyP gene by a variety of techniques. Genomic DNA used for the diagnosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for detection of a specific sequence or may be PCR amplified prior to analysis. RNA or cDNA may also be used. To detect a specific DNA sequence hybridization using specific oligonucleotides, direct DNA sequencing, restriction enzyme digest, RNase protection, chemical cleavage, and ligase-mediated detection are all methods which can be utilized. Oligonucleotides specific to mutant sequences can be chemically synthesized and labelled radioactively with isotopes, or non-radioactively using biotin tags, and hybridized to individual DNA samples immobilized on membranes or other solid-supports by dot-blot or transfer from gels after electrophoresis. The presence or absence of these mutant sequences are then visualized using methods such as autoradiography, fluorometry, or colorimetric reaction. Suitable PCR primers can be generated which are useful for example in amplifying portions of the subject sequence containing identified mutations.

Direct DNA sequencing reveals sequence differences between normal and mutant LyP DNA. Cloned DNA segments may be used as probes to detect specific DNA segments. PCR can be used to enhance the sensitivity of this method. PCR is an enzymatic amplification directed by sequence-specific primers, and involves repeated cycles of heat denaturation of the DNA, annealing of the complementary primers and extension of the annealed primer with a DNA polymerase. This results in an exponential increase of the target DNA.

Other nucleotide sequence amplification techniques may be used, such as

ligation-mediated PCR, anchored PCR and enzymatic amplification as would be understood by those skilled in the art.

Sequence alterations may also generate fortuitous restriction enzyme recognition sites which are revealed by the use of appropriate enzyme digestion followed by gel-blot hybridization. DNA fragments carrying the site (normal or mutant) are detected by their increase or reduction in size, or by the increase or decrease of corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme and the fragments of different sizes are visualized under UV light in the presence of ethidium bromide after gel electrophoresis.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. Small deletions may also be detected as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel electrophoresis. Alternatively, a single base substitution mutation may be detected based on differential primer length in PCR. The PCR products of the normal and mutant gene could be differentially detected in acrylamide gels.

Nuclease protection assays (S1 or ligase-mediated) also reveal sequence changes at specific locations. Alternatively, to confirm or detect a polymorphism restriction mapping changes ligated PCR, ASO, REF-SSCP and SSCP may be used. Both REF-SSCP and SSCP are mobility shift assays which are based upon the change in conformation due to mutations.

DNA fragments may also be visualized by methods in which the individual DNA samples are not immobilized on membranes. The probe and target sequences may be in solution or the probe sequence may be immobilized. Autoradiography, radioactive decay, spectrophotometry, and fluorometry may also be used to identify specific individual genotypes.

According to an embodiment of the invention, the portion of the DNA segment that is informative for a mutation, can be amplified using PCR. The DNA segment immediately surrounding a specific mutation acquired from peripheral blood or other tissue samples from an individual can be screened using constructed

oligonucleotide primers. This region would then be amplied by PCR, the products separated by electrophoresis, and transferred to membrane. Labelled probes are then hybridized to the DNA fragments and autoradiography performed.

In accordance with another embodiment of the present invention are antibodies which recognize epitopes within the LyP1 or LyP2 protein and which can be raised to provide information on the characteristics of the protein as well as for any mutant LyP1 or LyP2 protein. Generation of antibodies would enable the visualization of the protein in cells and tissues using Western blotting. In this technique, proteins are run on polyacrylamide gel and then transferred onto nitrocellulose membranes. These membranes are then incubated in the presence of the antibody (primary), then following washing are incubated to a secondary antibody which is used for detection of the protein-primary antibody complex. Following repeated washing, the entire complex is visualized using colourimetric or chemiluminescent methods.

Antibodies to the LyP1 or LyP2 protein also allow for the use of immunocytochemistry and immunofluorescence techniques in which the proteins can be visualized directly in cells and tissues. This is most helpful in order to establish the subcellular location of the protein and the tissue specificity of the protein.

In general, methods for the preparation of antibodies are well known (42). In order to prepare polyclonal antibodies, fusion proteins containing defined portions or all of the LyP1 or LyP2 protein or specific LyP1 or LyP2 generated mutants can be synthesized in bacteria by expression of corresponding DNA sequences in a suitable cloning vehicle. The protein can then be purified, coupled to a carrier protein and mixed with Freund's adjuvant (to help stimulate the antigenic response by the rabbits) and injected into rabbits or other laboratory animals. Alternatively, protein can be isolated from cultured cells expressing the protein. Following booster injections at bi-weekly intervals, the rabbits or other laboratory animals are then bled and the sera isolated. The sera can be used directly or purified prior to use, by affinity chromatography. The sera can then be used to probe protein extracts run on a polyacrylamide gel to identify the LyP1 or LyP2 protein or mutant protein. Alternatively, synthetic peptides can be made to the antigenic portions of these proteins and used to innoculate the animals.

To produce monoclonal LyP1 or LyP2 antibodies, cells actively expressing the

protein are cultured or isolated from tissues and the cell extracts isolated. The extracts or recombinant protein extracts, containing the LyP1 or LyP2 protein, are injected in Freund's adjuvant into mice. After being injected 9 times over a three week period, the mice spleens are removed and resuspended in phosphate buffered saline (PBS). The spleen cells serve as a source of lymphocytes, some of which are producing antibody of the appropriate specificity. These are then fused with a permanently growing myeloma partner cell, and the products of the fusion are plated into a number of tissue culture wells in the presence of a selective agent such as HAT. The wells are then screened to identify those containing cells making useful antibody by ELISA. These are then freshly plated. After a period of growth, these wells are again screened to identify antibody-producing cells. Several cloning procedures are carried out until over 90% of the wells contain single clones which are positive for antibody production. From this procedure a stable line of clones is established which produce the antibody. The monoclonal antibody can then be purified by affinity chromatography using Protein A Sepharose.

DrugScreening Assays

In accordance with another embodiment of the present invention there is provided assays for screening upstream activators and downstream effectors of human LyP1 or LyP2 protein. Cell culture systems, both cell and cell-free, in which the human LyP gene has been transfected and is being expressed can be tested with a number of agents to identify specific activators and substrates of the phosphatase through labelled phosphorylation studies of the protein in which phosphatase activity is quantitated. Such assays are also useful in identifying the downstream protein effects of such activation and their effects on transcription of various genes. Mutant forms of the human LyP can also be transfected into cell lines in order to study which specifically constructed mutations in the gene sequence which lead to altered activation of the phosphatase and eventually lead to alterations in T cell activation and differentiation.

Such culture systems enable the screening for pharmacological agents which will prevent activation of both normal and abnormally activated LyP as well as agents which will inhibit downstream cellular effects of the activated LyP1 and LyP2

protein. Transfected culture systems also permit for the identification of phosphatase inhibitors which will be useful as therapeutic compositions for treating lymphoid cell disorders.

Therapies

An important aspect of the biochemical studies using the genetic information of this invention is the development of therapies to circumvent or overcome the effect of abnormally activated LyP gene product, and thus prevent, treat, control serious symptoms or cure lymphoid cell associated disease. In view of the expression of the Lyp gene in a variety of lymphoid tissues one has to recognize that abnormally activated LyP may lead to a variety of lymphoid cell disorders such as leukemia and autoimmune disorders. Hence, in considering various therapies, it is understood that such therapies may be targeted at various lymphoid tissues where LyP1 or LyP2 is abnormally expressed.

In accordance with another embodiment of the present invention there is provided gene therapy as another potential therapeutic approach in which normal copies of the LyP gene are introduced into patients to successfully code for normal LyP1 or LyP2 protein in several different affected cell types. Mutated copies of the LyP gene in which the protein product is inactivated can also be introduced into patients. The gene must be delivered to those cells in a form in which it can be taken up and code for sufficient protein to provide effective function. Alternatively, in some mutants it has been possible to prevent disease by introducing another copy of the homologous gene bearing a second mutation in that gene or to alter the mutation, or use another gene to block its effect.

Retroviral vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression. The targeted cells however must be able to divide and the expression of the levels of normal protein should be high. The full length LyP gene can be cloned into a retroviral vector and driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest (such as lymphoid cells).

Other viral vectors which can be used include adeno-associated virus, vaccinia

virus, bovine papilloma virus, or a herpesvirus such as Epstein-Barr virus.

Gene transfer could also be achieved using non-viral means requiring infection *in vitro*. This would include calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes may also be potentially beneficial for delivery of DNA into a cell. Although these methods are available, many of these are lower efficiency.

Transplantation of normal genes or mutated genes which code for an inactive LyP1 or LyP2 into a targetted affected area of the patient can also be useful therapy for leukemia or associated malignancies. In this procedure, a LyP gene is transferred into a cultivatable cell type such as lymphoid cells, either exogenously or endogenously to the patient. These cells are then injected serotologically into the disease affected tissue(s).

The invention also provides a method for reversing a transformed phenotype resulting from the abnormal expression of the LyP human gene sequence and/or hyperactivation of a LyP1 or LyP2 protein product which is responsible for transformation of cells into a malignant phenotype such as leukemia. Antisense based strategies can be employed to explore gene function, inhibit gene function and as a basis for therapeutic drug design. The principle is based on the hypothesis that sequence specific suppression of gene expression can be achieved by intracellular hybridization between mRNA and a complementary anti-sense species. It is possible to synthesize anti-sense strand nucleotides that bind the sense strand of RNA or DNA with a high degree of specificity. The formation of a hybrid RNA duplex may interfere with the processing/transport/translation and/or stability of a target mRNA.

Hybridization is required for an antisense effect to occur. Antisense effects have been described using a variety of approaches including the use of antisense oligonucleotides, injection of antisense RNA, DNA and transfection of antisense RNA expression vectors.

Therapeutic antisense nucleotides can be made as oligonucleotides or expressed nucleotides. Oligonucleotides are short single strands of DNA which are usually 15 to 20 nucleic acid bases long. Expressed nucleotides are made by an expression vector such as an adenoviral, retroviral or plasmid vector. The vector is administered to the cells in culture, or to a patient, whose cells then make the antisense nucleotide. Expression vectors can be designed to produce antisense RNA,

which can vary in length from a few dozen bases to several thousand.

Antisense effects can be induced by control (sense) sequences. The extent of phenotypic changes are highly variable. Phenotypic effects induced by antisense are based on changes in criteria such as biological endpoints, protein levels, protein activation measurement and target mRNA levels.

Multidrug resistance is a useful model for the study of molecular events associated with phenotypic changes due to antisense effects since the MDR phenotype can be established by expression of a single gene mdr1 (MDR gene) encoding P-glycoprotein (a 170 kDa membrane glycoprotein, ATP-dependent efflux pump).

In the present invention, mammalian cells in which the LyP human cDNA has been transfected and which express an abnormal phenotype, can be additionally transfected with anti-sense LyP (LyP1 or LyP2) nucleotide DNA sequences which hybridize to the LyP gene in order to inhibit the transcription of the gene and reverse or reduce the abnormal phenotype. Alternatively, portions of the LyP gene can be targeted with an anti-sense LyP sequence specific for the kinase domains or the unique amino terminal sequence which may be responsible for the malignant phenotype. Expression vectors can be used as a model for anti-sense gene therapy to target the LyP which is expressed in abnormal cells. In this manner abnormal cells and tissues can be targeted while allowing healthy cells to survive. This may prove to be an effective treatment for cell abnormalities induced by LyP1 or LyP2.

Immunotherapy is also possible for the treatment of certain lymphoid cell disease. Antibodies can be raised to a hyperactive LyP1 or LyP2 protein (or portion thereof) and then be administered to bind or block the abnormal protein and its deliterious effects. Simultaneously, expression of the normal protein product could be encouraged. Administration could be in the form of a one time immunogenic preparation or vaccine immunization. An immunogenic composition may be prepared as injectables, as liquid solutions or emulsions. The LyP protein may be mixed with pharmaceutically acceptable excipients compatible with the protein. Such excipients may include water, saline, dextrose, glycerol, ethanol and combinations thereof. The immunogenic composition and vaccine may further contain auxiliarry substances such as emulsifying agents or adjuvants to enhance effectiveness. Immunogenic compositions and vaccines may be administered parenterally by injection

subcutaneously or intramuscularly.

The immunogenic preparations and vaccines are administered in such amount as will be therapeutically effective, protective and immunogenic. Dosage depends on the route of administration and will vary according to the size of the host.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in the form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Examples

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Methods of molecular genetics, protein and peptide biochemistry and immunology referred to but not explicitly described in this disclosure and examples are reported in the scientific literature and are well known to those skilled in the art.

Example 1 - Isolation of Novel Human PTPases, LyP1 and LyP2

To identify new members of the PTPase gene family that are expressed in thymocytes, a PCT-based approach was used with degenerate oligonucleotides directed at conserved regions of the PTPase catalytic domain. A fragment of ~400bp was amplified from thymocyte cDNA and identified PCR amplified clones corresponding to seven different phosphatases. Six clones were identical to previously isolated human phosphatases: PTP-PEST,[24] PTP1B,[25] TCPTP,[26] HPTPδ,[6] CD45 and PTPMEG2.[27] A seventh clone had no human homologue but was 90% homologous to the murine phosphatase Z70PEP.[10] This clone was used to screen a human thymocyte cDNA library. The first screening isolated two overlapping clones, P1 and P2 (Fig.1). Clone P2 was used to isolate a further three overlapping clones P3-P5 from the cDNA library. Assembly of the five overlapping clones revealed a

single cDNA of 2300bp containing an open reading frame (ORF) of 2076bp, predicting a protein of 692 amino acids. The sequence surrounding the putative ATG/methionine start codon contained a purine (A) at position -3 and G at +4, both regarded as important criteria for an eucaryotic initiation site.[32] The N-terminal region of the amino acid sequence (Fig 2) contained a single PTPase catalytic domain characterized by the conserved sequence motif (I/V)HCXXGXXRS/T. This sequence, thought to form the phosphate binding pocket for substrate, is found in all PTPases and is essential for their enzymatic activity. In addition to the 5 overlapping clones a single kb clone was isolated (P6, Fig. 1), with 200bp of its 5'-end overlapping nucleotides 1950-2055 of the complete cDNA previously isolated. However this was followed by an alternative 700bp, coding for an ORF totalling 2424bp. The long (3056bp) and short (2356bp) forms share nucleotides 1-2097 but code for alternative C-terminal sequence. These forms are designated LyPI and LyP2 respectively. LyP2 is an alternative spliced isoform of LyPI.

Polymerase chain reaction and subcloning of the PTPases clones.

Total RNA was prepared from thymocytes using Trizol reagent (Gibco-BRL). First strand cDNA synthesis was performed with oligo-dt primer and Superscript II RT (Gibco-BRL). This was used as a template for PCR amplification with Taq DNA polymerase (Perkin Elmer Cetus) and the following degenerate primers:

PTP1-GCGGATCCTCIGA(C/T)TA(C/T)AT(A/C/T)AA(T/C)GC [sense, SEQ. ID NO: 5]

PTP2-GCGAATTCCCIACICCIGC(A/G)CT(G/A)CA(G/A)TG [antisense, SEQ. ID NO:6].

These degenerate primers are designed to match two highly conserved sequences within PTPase catalytic domains, XDYINA and HCSAG/VG respectively. PCR was performed as follows: five cycles of 60sec. at 94°C, 30 sec. at 37°C and 60 sec. at 72°C , and a further 25 cycles with an annealing temperature of 45°C. Fragments of ~400bp were isolated, cloned and sequenced.

Isolation and Sequencing of LyP1 and LyP2 cDNA clones.

A cDNA library from human thymocytes was screened with a [^{32}P] labelled 430bp LYPI fragment obtained by PCR. Plaques were transferred to ICN Biotrans nylon filters and screened by hybridization at 65°C in 5 x SSC, 5 x Denhart's solution, O.1%SDS (22). Phage DNA was prepared from positive plaques, cDNA inserts were excised, subcloned into pUC-19, and sequenced. To obtain the complete LyP1 cDNA, secondary and tertiary library screenings were performed with the 1.3kb and 0.6kb partial cDNA clones isolated in the first screening (Fig. 1) . One clone (P5) from the second screening was found to contain the carboxyterminal sequence of the spliced form of LyP1 (LyP2).

Example 2 - LyP2 is produced by alternative RNA splicing of the LyP1 message.

To confirm the hypothesis that LyP2 was produced by alternative splicing of LyP1 RNA, three oligonucleotides matching sequences around the putative splicing sites were used in PCR amplifications on a genomic DNA template (Fig.3A). Oligonucleotide 1 corresponded to the common nucleotides 2076-2097 of LyP1 and LyP2 (Fig 3), oligonucleotide 2 to LyP2 untranslated area adjacent to the stop codon

(nucleotides 2150-2168), and olignucleotide 3 to LyP1 sequence immediately downstream of primer 1 (nucleotides 2098-2120). The resultant PCR products are shown in Fig 3B. PCR amplification with primers 1 and 3 created an approximately 3.5kb DNA fragment, suggesting the presence of an intron between the primers. However PCR with primers 1 and 2 resulted in a much smaller fragment of 100bp, the size expected from LyP2 cDNA sequence. Upon sequencing, the 5' end sequence of the 3.5kb fragment was found to contain the alternative C-terminus, stop codon and untranslated nucleotide sequence of LyP2 (Fig 3C). This clearly demonstrated that LyP1 and LyP2 are the alternatively spliced transcripts of a single gene. While the 3.5kb intron is spliced out of the LYP1 form, this does not occur in the LyP2 isoform and as a result only 7 amino acids are added and an alternative stop codon is utilized.

Example 3 - Characterization of LyP1 and LyP2 proteins

To determine whether LyPI and LyP2 proteins are expressed at their predicted sizes or undergo processing in eukaryotic cells, the full length cDNAs were tagged at their 5' end with a haemagglutinin (HA) epitope and transfected into Cos-7 cells. The cDNAs of LyPI and LyP2 code for polypeptides of molecular weight (Mw) 92,000 and 78,000 respectively. On SDS-PAGE gel the molecular weights of the transfected proteins were close to the predicted values (Fig.5). Antibodies to the HA tag recognized a single protein with an apparent Mw of 96kDa in LyPI transfected cells and a single protein with an apparent molecular Mw of 80kDa in LyP2 transfectants, indicating that these phosphatases do not undergo significant post translation modifications.

Preferential Expression of LyP1 transcripts in Hematopoietic Tissues and Cells

Northern blot analysis of mRNA from various human tissues using a LyPI cDNA probe revealed a major transcript of approximately 3.8kb in all of the lymphoid tissues examined (Fig.4). Substantial levels of LyP mRNA were detected in spleen, lymph node, peripheral leukocytes, tonsil B and T lymphocytes, and to a lesser degree in bone marrow. In contrast, LyP transcripts were not detected in prostate, ovary, testis fetal-liver or colon tissues. A low level of LyP expression could however be detected in the small intestine and appendix mucosa, presumably due to the presence of contaminating lymphocytes. LyP2 expression could not detected by Northern blot

analysis using a probe to the last 21 specific nucleotides. Therefore its expression relative to LyPI was quantified by competitive PCR on polyA+-derived single strand thymocyte cDNA. The internal standards were constructed by deleting 140bp from both LyP1 and LyP2 cDNAs. Co-amplification of the target cDNA with various concentration of internal control revealed that a concentration of 0.05×10^{-4} pM control DNA was needed to produce equivalent amounts of LyP2 target and control PCR product while 5×10^{-4} PM of internal standard was required with specific primers to LyP1 (Fig.6). These results suggest that the level of LyP1 expression in thymocytes is approximately 100 fold greater than the level of LyP2 expression.

Northern blot analysis of LyP1 and LyP2 RNA.

Total RNA was extracted from thymocytes using Trizol reagent (Gibco BRL). Poly A+ RNA was isolated by two passages through an oligo(dt) column. 2μg of Poly A+ RNA per sample was electrophoresed in a 1% agarose formaldehyde gel and capillary blotted onto nitrocellulose filters. Filters and a human multiple tissue poly A+ RNA northern blots (Clontech) were hybridized overnight at 42°C with [^{32}p] labelled LyP cDNA probes in 50% formamide, 5 x SSC, 5 x Denhart's solution, 0.1% SDS, 50μl Na_2HP0_4 pH 6.5, and denatured Salmon sperm DNA (100μg/ml). Specifically, 2μg of poly A^+ RNA from various human tissues was hybridized with a 1.3 kb cDNA probe common to both LyP1 and LyP2 and exposed 7 days or 24 hr with Actin. After hybridization the final wash was performed in 0.2%SSC, O. 1%SDS at 55°C (22).

Relative Quantification of LyP1 and LyP2 mRNA by Competitive Polymerase Chain Reaction.

The relative levels of LyP1 and LyP2 messenger RNA (mRNA) in thymocytes were quantified by competitive PCR using a synthetic cDNA as internal standard. This technique involves co-amplification of a target cDNA (produced from the corresponding mRNA by reverse transcription) and of the internal standard. The target cDNA and the internal standard use the same primer sequence, but yield PCR products of different sizes that can be resolved on gel electrophoresis. In the exponential phase of the amplification, the amount of target cDNA can be quantified by comparison with the amplification of various amounts of the internal standard. The amount of target

sequence in the sample is estimated by the amount of control producing an equivalent amounts of PCR products. The internal standards were constructed by deleting 140bp from both LyP1 and LyP2 cDNAs, using two EcoRl sites found in position 1805 and 1945. PCR primers: The 5' primer for both LyP1 and LyP2- corresponds to nucleotides 1660-1682 with the 3' primer for LyPl- corresponding to nucleotides 2425-2447, while the 3' primer for LyP2- corresponds to nucleotides 2075-2097. cDNA was prepared from oligo (dT) selected mRNA as described previously. Aliquots of thymus cDNA were co amplified with varying amounts of internal standard for 26 cycles for LyP1 and 35 cycles for LyP2. (denaturating 94°C 30 sec., annealing at 54°C and elongation 45 sec. at 72°C). The PCR products (40µl) were electrophoresed on 1.2% agarose gel, stained with ethidium bromide and photographed. The possibility of genomic DNA contamination in the RT PCR reaction was excluded with the appropriate controls.

Example 4 - Cellular localization of LyP1 and LyP2 in Transfected Cos-7 cells

In order to determine the cellular localization of the two phosphatases, the distribution of both LyP1 and LyP2 was determined by indirect immunofluorescence in transiently transfected Cos-7 cells. An HA epitope was attached as a three tandem repeat to 5' end of both LyP1 and LyP2 cDNAs in the pCDNA3 eukaryotic expression vector. 48 hours after transfection , cells were fixed and incubated with antibodies to the HA epitope. Cos-7 cells transfected with either LyP1 and LyP2 displayed prominent perinuclear and cytoplasmatic staining but no staining of the nucleus (Fig. 7). No fluorescence was noted in Cos-7 cells transfected with vector alone. The pattern of staining suggests that both of these phosphatases are predominantly cytoplasmatic.

Cell preparation and cell lines.

Thymuses were obtained from children undergoing open heart surgery. Mononuclear cells were isolated by Ficoll-Hypaque gradient. Adherent cells were removed by incubation to plastic dishes for 60 minutes at 37°C. The resulting thymocytes are typically >95% CD3+. Lymphocytes were isolated from tonsil tissue by Ficoll-Hypaque centrifugation, following by rosetting with neuraminidase treated sheep red blood cells (RBC) to isolate T lymphocytes. After isolating rosettes by Ficoll-Hypaque gradient centrifugation, T cells were released with ACT treatment (0.75%

NH_4Cl in 20 mmol/L Tris, pH 7.2) of the rosettes to lyse the red blood cells. The buffy layer, containing the B cells, was washed three times with PBS. The resultant T lymphocytes are typically 98% to 99% CD3+and the B lymphocytes are typically 97% to 98.5 % CD 19+.

Transfection

To examine the actual size of the expressed proteins LyP1 and LyP2, cDNAs were inserted into the pCDNA3 eukaryotic expression vector (Invitrogen). An HA epitope (YPYDVPDYA) derived from the haemagglutinin protein of influenza virus, was inserted as three-tandem repeat at the 5' end of the coding sequences of both LyP forms. The constructs were verified by sequencing. Cos-7 cells were transfected with 5µg DNA and 50µl of Lipofectamine (Gibco -BRL) for 5 hours according to the manufacture's instructions. 24 hours before transfection 0.5 $x10^{-6}$ Cos-7 cells were plated on 60mm plates in Dulbaco's modified Eagle medium (DMEM) containing 10% fetal calf serum. To examine the cellular localization of the expressed proteins, Cos-7 cells were transfected with 2µg DNA and 17µl of Lipofectamine for 5 hours, incubated on sterile cover slips in six well plates (0.3 x 10^{-6} /plate) in DMEM containing 10% fetal calf serum for 48 hours and stained.

Western Blotting .

Cos-7 cells (0.5x 10^{-6} /plate) were washed three times with cold PBS and solubilized in cold lysis buffer (2OmM Tris pH 7.5, l5OmM NaCl, 1mM EDTA, 1% NP-40, 1mM PMSF). The lysates were cleared by centrifugation. SDS sample buffer was added to the clarified lysates and resolved by 7% SDS-PAGE . The proteins were electrophoretically transferred to Hybond-C super nitrocellulose membrane (Amersham Life Science). Membranes were blocked with 5% non fat milk and probed with HA monoclonal antibody from Bleo-Berkely. Detection was performed with horseradish peroxidase conjugated sheep anti mouse purchased from (Amersham Life Science) and chemiluminescence reagent from Kirkeggard &Perry laboratories.

Indirect Immunofluorescence

48 hours post transfection Cos-7 cells were washed in PBS and fixed for 30 min at room temperature in 2% paraformaldehyde. Cell permeabilization was performed with 0.1% Triton X100. After blocking non-specific sites with 5% Donkey serum, the cells were incubated for 60 min at room temperature with monoclonal anti HA tag (1:1000 in PBS) from Blco-Berkely. The cells were washed and exposed for 45 min. to cy3 conjugated affinipure Donkey anti mouse IgG (1:1000 in PBS) from Jackson Immunoresearch Laboratories, Inc. After 3-4 washes immunoreactivity was detected by fluorescence microscopy.

Example 5 - Identifying the Chromosomal Location of Lyp

A 1.8 kb LyP cDNA fragment was used as a probe to examine the chromosomal location of LyP. Biotynylated LyP probe was prepared by nick translation for fluorescence *in situ* (FISH) to normal human lymphocyte chromosomes (counterstained with propidium iodide and 4',6-diamidin-3-phenylindol-dihydrochloride, DAPI, according to published methods (43, 44)). The probe was detected with avidin-FITC. Images of metaphase preparations were captured by thermoelectrically cooled charge coupled camera (Photometrics, Tucson, AZ). Separate images of DAPI banded chromosomes (45) and FITC targeted chromosomes were obtained and merged electronically using image analysis software (Yale University, New Haven, CT) and pseudo coloured blue (DAPI) and yellow (FITC) as described by Boyle et al., (44). The band assignment was determined by measuring the fractional chromosome length and by analyzing the banding pattern generated by the DAPI counterstained image (46).

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TGTTTCTTCAGCAGAACTGAATTATTCACTGCCATATGACTCTAAACACCAAATACGTAATGCC
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TTACAATTCACATAGTTCTTTATCACTGAATTCTCCAACCAATATTTCCTCACTATTGAACCAG
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CCTTCTTTCTTGCCGATGAAGATTGTATGCAGGCCCAATCTATAGAAACATATTCTACTAGCTA
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CACAAGGAGTAAGAGTTTGAAAATTTTGCGAAACATGAAAAAGAGTATCTGTAATTCTTGCCC
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GCAAACCGTTTTTCAAAACCCAAAGGACCAAGGAATCCACCACCAACTTGGAATATTTAATAA
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TAGTTAAAATAAGTGCTCTATATGCATAATATGAAGATATGCTAATGTGTTAATAGCTTTTAAA
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TTGAATGTAACAAG

Table 1: Human LyPl cDNA sequence

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GAAGCTGAAAAGGCAATCTACCAAGTACAAGGCAGACAAAACCTATCCTACAACTGTGGCTG
AGAATGCCAAGAATATCAAGAAAAACAGATATAAGGATATTTTGCCCTATGATTATAGCCGGG
TAGAACTATCCCTGATAACCTCTGATGAGGATTCCAGCTACATCAATGCCAACTTCATTAAGG
GAGTTTATGGACCCAAGGCTTATATTGCCACCCAGGGTCCTTTATCTACAACCCTCCTGGACTT
CTGGAGGATGATTTGGGAATATAGTGTCCTTATCATTGTTATGGCATGCATGGAGTATGAAAT
GGGAAAGAAAAAGTGTGAGCGCTACTGGGCTGAGCCAGGAGAGATGCAGCTGGAATTTGGCC
CTTTCTCTGTATCCTGTGAAGCTGAAAAAAGGAAATCTGATTATATAATCAGGACTCTAAAAG
TTAAGTTCAATAGTGAAACTCGAACTATCTACCAGTTTCATTACAAGAATTGGCCAGACCATG
ATGTACCTTCATCTATAGACCCTATTCTTGAGCTCATCTGGGATGTACGTTGTTACCAAGAGGA
TGACAGTGTTCCCATATGCATTCACTGCAGTGCTGGCTGTGGAAGGACTGGTGTTATTTGTGCT
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GAGTCAGCTGTACTAGCAACTGCTCCAAGGATAGATGATGAAATCCCCCCTCCACTTCCTGTA
CGGACACCTGAATCATTTATTGTGGTTGAGGAAGCTGGAGAATTCTCACCAAATGTTCCCAAA
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AAATCAGGTAAAAATTTCTCTTGGCTTTAGATGACATTTAGCCCTAAGATTGGAAGAATGGTT
CGTTAAGTTTAGAGTAATTCACTTCAGGAAGTTACTTGGTTCCCATAATAGCTTCCAGTATTCA
TTGATTTATTTCTGGCTTTCCCAGACTAGAAATTTTGTAAAGAGTCATGGGGGAAGCTAGGGCT
AACCAGAAAATAAAATAAAAATAATGGGATAAAAAATCGGAACTACTGTTTTCCCCCTAGTCG
GAGCACATCCGG

Table 2: Human LyP2 cDNA sequence

MDQREILQKFLDEAQSKKITKEEFANEFLKLKRQSTKYKADKTYPTTVAENAKNIKKNRYKDILPY
DYSRVELSLITSDEDSSYINANFIKGVYGPKAYIATQGPLSTTLLDFWRMIWEYSVLIIVMACMEYE
MGKKKCERYWAEPGEMQLEFGPFSVSCEAEKRKSDYIIRTLKVKFNSETRTIYQFHYKNWPDHDV
PSSIDPILELIWDVRCYQEDDSVPICIHCSAGCGRTGVICAIVDYTWMLLKDGIIPENFSVFSLIREMR
TQRPSLVQTQEQYELVYNAVLELFKRQMDVIRDKHSGTESQAKHCIPEKNHTLQADSYSPNLPKST
TKAAKMMNQQRTKMEIKESSSFDFRTSEISAKEELVLHPAKSSTSFDFLELNYSFDKNADTTMKW
QTKAFPIVGEPLQKHQSLDLGSLLFEGCSNSKPVNAAGRYFNSKVPITRTKSTPFELIQQRETKEVDS
KENFSYLESQPHDSCFVEMQAQKVMHVSSAELNYSLPYDSKHQIRNASNVKHHDSSALGVYSYIP
LVENPYFSSWPPSGTSSKMSLDLPEKQDGTVFPSSLLPTSSTSLFSYYNSHSSLSLNSPTNISSLLNQE
SAVLATAPRIDDEIPPPLPVRTPESFIVVEEAGEFSPNVPKSLSSAVKVKIGTSLEWGGTSEPKKFDDS
VILRPSKSVKLRSPKSELHQDRSSPPPPLPERTLESFFLADEDCMQAQSIETYSTSYPDTMENSTSSK
QTLKTPGKSFTRSKSLKILRNMKKSICNSCPPNKPAESVQSNNSSSFLNFGFANRFSKPKGPRNPPPT
WNI

Table 3: LyPl Amino Acid Sequence

MDQREILQKFLDEAQSKKITKEEFANEFLKLKRQSTKYKADKTYPTTVAENAKNIKKNRYKDILPY
DYSRVELSLITSDEDSSYINANFIKGVYGPKAYIATQGPLSTTLLDFWRMIWEYSVLIVMACMEYE
MGKKKCERYWAEPGEMQLEFGPFSVSCEAEKRKSDYIIRTLKVKFNSETRTIYQFHYKNWPDHDV
PSSIDPILELIWDVRCYQEDDSVPICIHCSAGCGRTGVICAIVDYTWMLLKDGIIPENFSVFSLIREMR
TQRPSLVQTQEQYELVYNAVLELFKRQMDVIRDKHSGTESQAKHCIPEKNHTLQADSYSPNLPKST
TKAAKMMNQQRTKMEIKESSSFDFRTSEISAKEELVLHPAKSSTSFDFLELNYSFDKNADTTMKW
QTKAFPIVGEPLQKHQSLDLGSLLFEGCSNSKPVNAAGRYFNSKVPITRTKSTPFELIQQRETKEVDS
KENFSYLESQPHDSCFVEMQAQKVMHVSSAELNYSLPYDSKHQIRNASNVKHHDSSALGVYSYIP
LVENPYFSSWPPSGTSSKMSLDLPEKQDGTVFPSSLLPTSSTSLFSYYNSDSSLSLNSPTNISSLLNQE
SAVLATAPRIDDEIPPPLPVRTPESFIVVEEAGEFSPNVPKSLSSAVKVKIGTSLEWGGTSEPKKFDDS
VILRPSKSVKLRSPKSGKNFSWL

Table 4: LyP2 Amino Acid Sequence

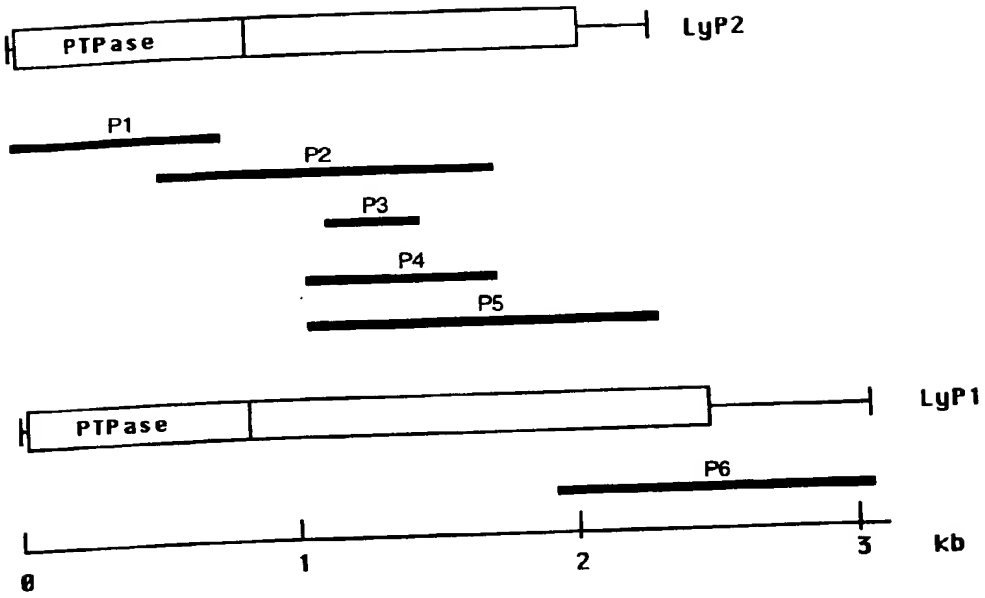

FIGURE 1

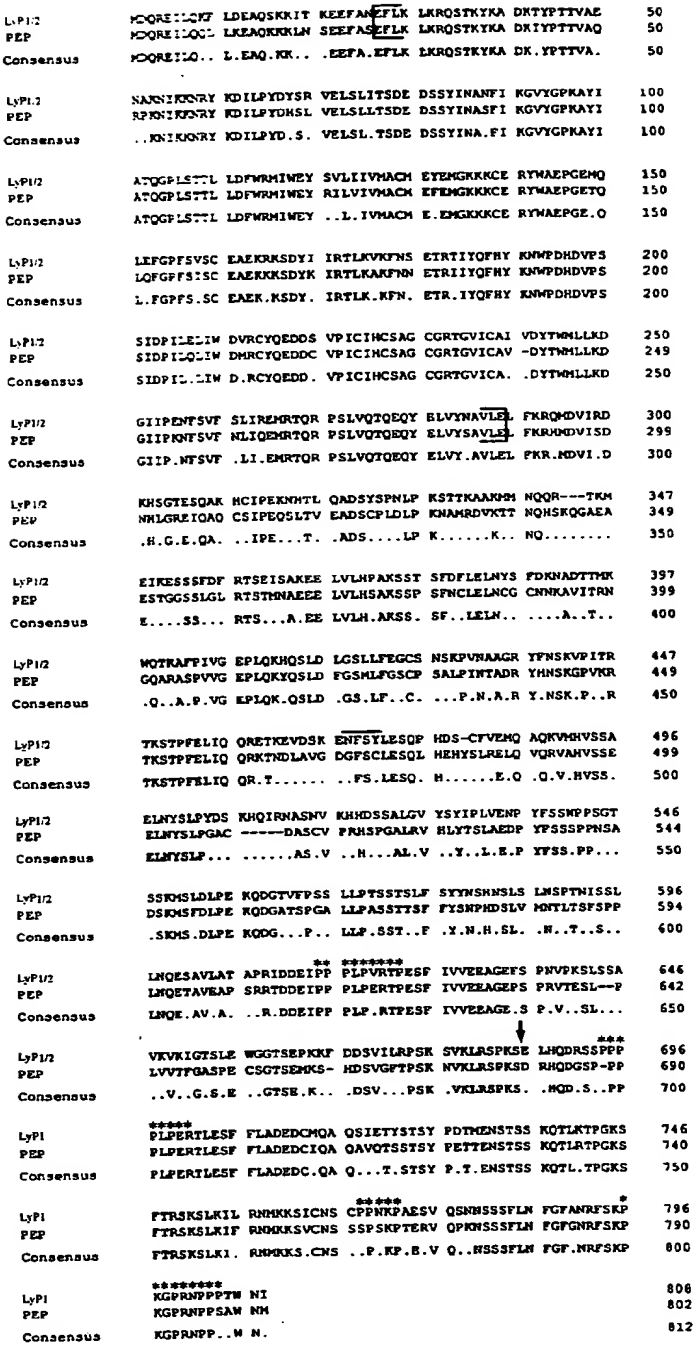

FIGURE 2

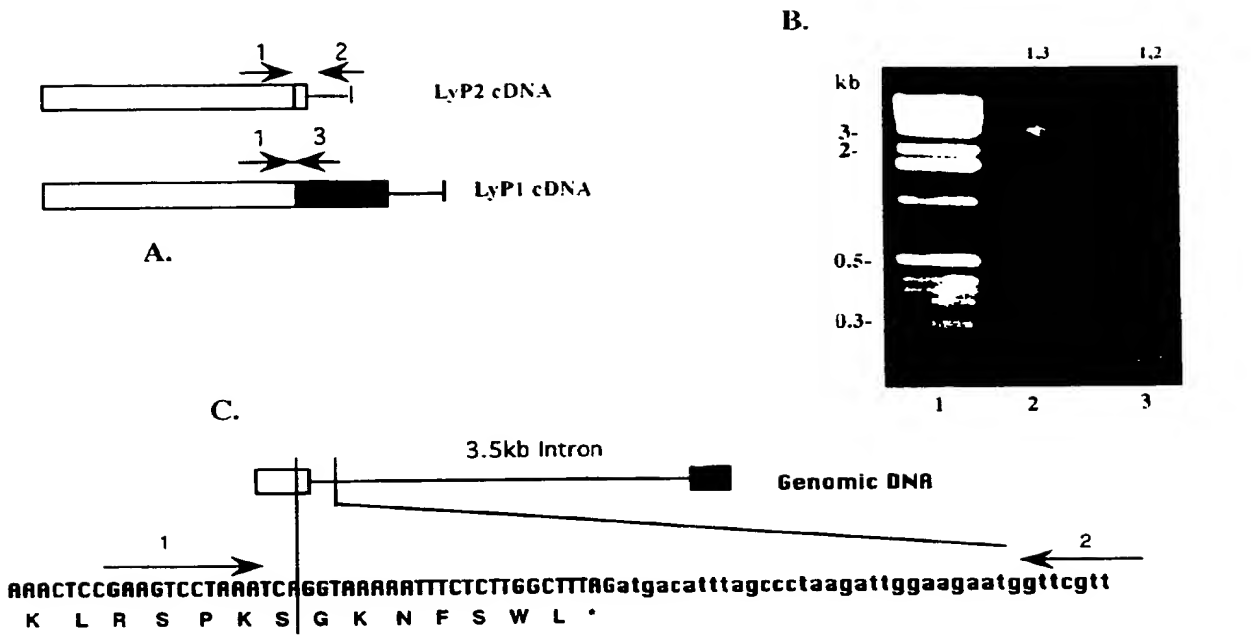
1
2
LyP2 cDNA
1
3
LyP1 cDNA
A.
B.
1.3
1.2
kb
3-
2-
0.5-
0.3-
1
2
3
C.
3.5kb Intron
Genomic DNA
1
2
AAACTCCGAAGTCCTAAATCAGGTAAAAATTTCTCTTGGCTTTAGatgacatttagccctaagattggaagaatggttcgtt
K L R S P K S G K N F S W L *

FIGURE 3

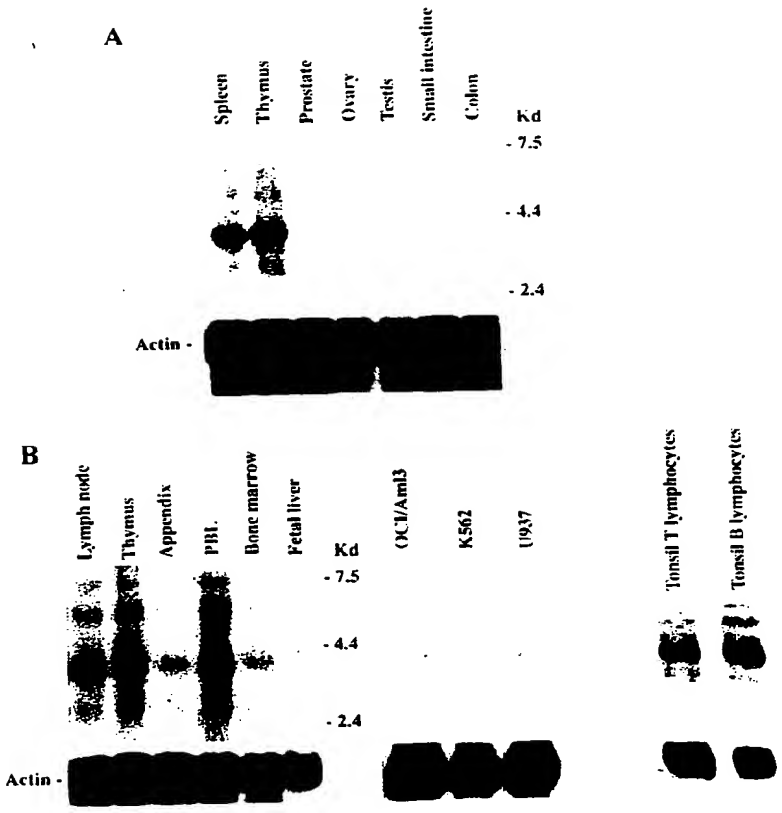
A
Spleen
Thymus
Prostate
Ovary
Testis
Small intestine
Colon
Kd
- 7.5
- 4.4
- 2.4
Actin -
B
Lymph node
Thymus
Appendix
PBL
Bone marrow
Fetal liver
Kd
- 7.5
- 4.4
- 2.4
OCI/Aml3
K562
U937
Tonsil T lymphocytes
Tonsil B lymphocytes
Actin -

FIGURE 4

FIGURE 5

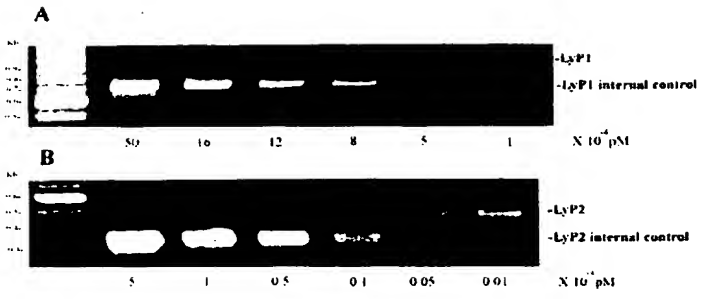
A
-LyP1
-LyP1 internal control
50
10
12
8
5
1
X 10⁻⁴ pM
B
-LyP2
-LyP2 internal control
5
1
0.5
0.1
0.05
0.01
X 10⁻⁴ pM

FIGURE 6

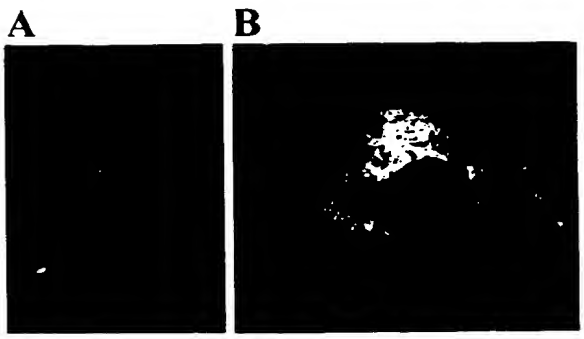

FIGURE 7

FIGURE 8